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# *In vitro* somatic embryogenesis of high yielding varieties of rice (*Oryza sativa* L.)

**Subhadra Rani Mallick, Divya Gautam and Gyana Ranjan Rout***

Department of Agricultural Biotechnology, College of Agriculture, Orissa University of Agriculture and Technology, Bhubaneswar- 751 003, Odisha, India.

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**Rice (*Oryza sativa* L.) belongs to the family Gramineae and is the staple food for half of the world's population and occupies almost one-fifth of the total land area covered under cereals. Now-a-days, the production of rice is hampered due to climatic changes. Therefore, it is essential to develop variety which is tolerant to abiotic and biotic stresses. The present investigation was conducted to establish an efficient and simple protocol for regeneration of four agronomically important *indica* rice varieties (Khandagiri, Udayagiri, Swarna and Pratikhya). Somatic embryogenesis were achieved from immature zygotic embryos on Murashige and Skoog (MS) medium supplemented with 3 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid), 1.0 mg/l kinetin and 3% (w/v) sucrose within 4 weeks of culture. The secondary somatic embryogenesis was also achieved in subsequent subculture on MS medium supplemented with 2 mg/l 2,4-D and 2.0 mg/l kinetin and 200 mg/l L-proline. The percentage of embryogenic calli proliferation were 82.4, 83.7, 88.4 and 84.4 in variety Khandagiri, Udayagiri, Swarna and Pratikhya respectively on MS basal medium supplemented with 3.0 mg/l 2,4-D, 2.0 mg/l Kinetin and 200 mg/l L-proline. Inclusion of higher concentration of L-proline (400 mg/l) in the induction medium, the growth of calli was reduced. The maximum percentage of somatic embryo germination took place in medium supplemented with 2.0 mg/l kinetin, 0.25 mg/l NAA and 50 mg/l adenine sulfate within 4 weeks of culture. The regenerated plantlets were transferred to pots for acclimatization. About 80% of plants were survived in the greenhouse condition.**

**Key words:** Somatic embryogenesis, immature zygotic embryos, *Indica* rice, plant regeneration.

## INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops of family Gramineae cultivated for more than 10,000 years (Sasaki, 2005). Currently, this crop supports more than 50% of the world population (Christou, 1997). Rice consumers are increasing at the rate of 1.8% every year (FAO/STAT, 2001). But the rate of growth in rice production has slowed down. It is estimated that rice production has to be increased 50% by 2025 (Khush and Virk, 2000). The available cultivable land is being reduced day by day due to unplanned industrialization and population growth. Both abiotic and biotic stresses making a great impact for reduction of production. Thus, there is a constant need to improve crops to overcome all these hazards. Resistant varieties have to be developed to meet the demand. Induction of tolerance requires an efficient breeding system. *In vitro* system is an alternative method for genetic improvement of rice. Efficient plant regeneration system leads to success in the crop improve-

*Coressponding author. E-mail: grrout@rediffmail.com.

ment through genetic transformation. It is often difficult to establish embryogenic cell cultures and to regenerate plants from these cultured cells specially those belonging to *Indica* subspecies (Jain, 1997). Even within the *Indica* group, there are significant variations in the *in vitro* culture responses among the different genotypes (Khanna and Raina, 1998). The recalcitrant nature of this subspecies has, in fact, been a major limiting factor in the transfer of available useful genes (Toenniessen, 1991). Therefore, the identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration *in vitro* are key steps in rice genetic improvement program through application of biotechnology (Hoque and Mansfield, 2004).

Some of the notable information on transgenic indica rice are reported (Lin and Zhang, 2005; Karim et al., 2007; Yang et al., 2010, 2012). This present investigation deals with the development of efficient protocol in upland (Khandagiri and Udayagiri), lowland (Swarna and Pratikhya) indica rice varieties which is suitable for genetic transformation experiment.

## MATERIALS AND METHODS

### Plant materials

Semi-mature seeds of *O. sativa* vars. Khandagiri and Udayagiri (upland) and Swarna and Pratikhya (lowland) were collected from rice germplasm centre of Orissa University of Agriculture and Technology, Bhubaneswar, India. The seeds were washed with 2% bavistin (w/v) for 30 min, dehusked and surface sterilized with 70% ethanol for 2 min followed by washing twice with sterilized distilled water with a drop of Tween 20 with continuous shaking for 15 min. Further, the seeds were treated with 0.2% (w/v) $HgCl_2$ solution for 5 min followed by rinsing 4 to 5 times with sterile distilled water and blot dry on sterilized filter paper. Immature embryos were aseptically cultured on MS (Murashige and Skoog, 1962) medium supplemented with various concentrations of benzyl aminopurine (BA) or kinetin (Kn) (0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/l), naphthalene acetic acid (NAA) or 2, 4-D (0, 0.5, 1.0, 1.5, 2.0, 3.0 mg/l) alone or in combinations for embryogenic callus culture. The pH of the media was adjusted to 5.7 using 0.1N NaOH or 0.1N HCl prior to addition of 0.8% (w/v) agar (Himedia, India). Routinely, 20 ml of molten medium was dispensed into 25 × 150 mm glass test tubes (Borosil, India), capped with non-absorbent cotton plugs. The cultures were sterilized at 121°C and 15 psi for 15 min. The cultures were incubated under 16 h photoperiod and fully dark at 25 ± 2°C for 4 to 6 weeks.

### Induction of embryogenic calli

After four weeks of incubation, data were taken on total callus induction frequency (embryogenic and non-embryogenic). Only embryogenic calli were subcultured to fresh medium supplemented with different concentrations of BA or kinetin and 2,4-D or NAA (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) singly or in combinations for induction of somatic embryo. In another experiment, different concentrations of L-proline (0, 50, 100, 150, 200 and 300 mg/l) was added in the embryogenic culture medium to enhance the embryogenic potential. The cultures were incubated under dark at 25 ± 2°C. Subculturing was made every 4 week intervals. The media were solidified with 0.8% (w/w) agar-agar. Morphological changes were recorded through visual observations at every 4- week intervals. The embryogenic response and number of somatic embryos per culture were recorded. Each treatment had 20 replicates and the experiment was repeated three times. Data were also recorded in respect of embryogenic frequency, number of somatic embryos and frequency of normal embryos per culture. The data were analyzed statistically by the Duncan's multiple range test (Duncan, 1955). Means followed by the same letter within columns were not significantly different at $P < 0.05$.

### Germination of somatic embryos

The group of somatic embryos was transferred to MS basal medium supplemented with different concentration of growth regulators for maturation and germination. Regeneration medium was based on the formulation of MS basal salt supplemented with NAA 0.2 to 1.0 mg/l, Kn 1.0 to 4 mg/l, 30 g/l (w/v) sucrose and gelled with 8.0 g/l (w/v) agar. The pH of the medium was adjusted to 5.8 before sterilization. For regeneration, eight-week-old somatic embryos were placed into test tubes, each containing 15 ml regeneration medium. All such cultures were kept under 16 h photoperiod at 25 ± 2°C with 3000 lux light intensity. Data on percent of embryo germination/ culture, number of germinated plant/culture were statistically by the Duncan's multiple range test (Duncan, 1955).

### Acclimatization

About 250 somatic embryos derived plantlets were transferred to the earthen pots with soil mixture at the ratio of 1: 1: 1 (sand: soil: FYM) and kept in the greenhouse with 85% relative humidity. The watering was given at every two day intervals till new growth occurring in the plantlets.

## RESULTS AND DISCUSSION

Production of embryogenic calli with high regeneration capacity is a prerequisite for highly efficient transformation of rice. Many efforts have been made to identify suitable ex-plants in rice to induce embryogenic calli under appropriate culture condition. Different growth regulators like auxins and cytokinins were used to develop the embryogenic calli from semimature embryos of upland and lowland rice varieties. The medium devoid of growth regulators did not show any positive impact on callus development. The embryogenic calli were initiated on basal MS medium supplemented with 2.0 to 3.0 mg/l 2,4-D and 1.0 to 1.5 mg/l kinetin within 4 weeks of culture. The growth of the calli was depended on the genotypes tested. The cultures were incubated in the 16 h photoperiod did not show any growth of the calli rather it became dried. The cultures incubated in continuous dark for 4 to 8 weeks, the yellowish white, friable callus were developed. The maximum percentage of calli development was achieved in MS medium supplemented with 3.0 mg/l 2,4-D and 1.0 mg/l kinetin (Figure 1A). The percentage embryogenic calli development was 73.4, 74.7, 75.4 and 80.6 in variety Khandagiri, Udayagiri, Swarna and Pratikhya respectively (Table 1). Among the two cytokinins used, kinetin has significant impact on embryogenic calli development as compare with BA + 2,4-D. The medium having BA + NAA and Kn + NAA did

**Table 1.** Effect of auxins and cytokinins on embryogenic calli development from semimature zygotic embryos of *O. sativa* varieties Khandagiri, Udayagiri, Swarna and Pratikhya after 4 weeks of subculture.

| MS + growth regulator (mg/l) | | | | Development of embryogenic calli (%) [mean (average) ±SE]* | | | |
|---|---|---|---|---|---|---|---|
| **Kn** | **BA** | **2,4-D** | **NAA** | **Khandagiri** | **Udayagiri** | **Swarna** | **Pratikhya** |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0 | 0.25 | 0.5 | 0 | 12.3 ± 0.6$^{a}$ | 18.7 ± 0.5$^{b}$ | 15.1 ± 0.6$^{a}$ | 15.4 ± 0.6$^{a}$ |
| 0 | 0.25 | 1.0 | 0 | 18.2 ± 0.8$^{c}$ | 24.3 ± 0.6$^{c}$ | 22.2 ± 0.8$^{c}$ | 27.2 ± 0.8$^{c}$ |
| 0 | 0.25 | 2.0 | 0 | 33.6 ± 0.7$^{e}$ | 46.7 ± 0.8$^{f}$ | 45.6 ± 0.7$^{h}$ | 42.6 ± 0.7$^{f}$ |
| 0 | 0.5 | 2.0 | 0 | 46.8 ± 0.6$^{g}$ | 51.9± 0.5$^{h}$ | 46.8 ± 0.6$^{h}$ | 48.8 ± 0.6$^{g}$ |
| 0 | 0.5 | 3.0 | 0 | 51.2 ± 0.8$^{h}$ | 52.1 ± 0.9$^{h}$ | 51.2 ± 0.8$^{i}$ | 58.2 ± 0.5$^{i}$ |
| 0.25 | 0 | 1.0 | 0 | 38.4 ± 0.8$^{f}$ | 37.3 ± 0.7$^{e}$ | 38.4 ± 0.8$^{f}$ | 41.7 ± 0.6$^{f}$ |
| 0.50 | 0 | 2.0 | 0 | 46.2 ± 0.7$^{g}$ | 49.7 ± 0.6$^{g}$ | 46.2 ± 0.7$^{h}$ | 43.2 ± 0.9$^{f}$ |
| 0.50 | 0 | 3.0 | 0 | 66.6 ± 1.0$^{j}$ | 67.8 ± 1.0$^{j}$ | 66.6 ± 1.0$^{l}$ | 61.6 ± 1.0$^{j}$ |
| 1.0 | 0 | 3.0 | 0 | 73.4 ± 0.6$^{k}$ | 74.7 ± 0.8$^{k}$ | 75.4 ± 0.6$^{m}$ | 80.6 ± 0.6$^{k}$ |
| 0 | 0.25 | 0 | 1.0 | 15.6 ± 07$^{b}$ | 15.6 ± 0.7$^{a}$ | 19.6 ± 0.7$^{b}$ | 19.0 ± 0.7$^{b}$ |
| 0 | 0.25 | 0 | 1.0 | 26.4 ± 0.8$^{d}$ | 31.6 ± 0.6$^{d}$ | 30.4 ± 0.8$^{d}$ | 32.4 ± 0.7$^{d}$ |
| 0 | 0.5 | 0 | 3.0 | 34.4 ± 0.9$^{e}$ | 39.5 ± 0.6$^{e}$ | 34.4 ± 0.9$^{e}$ | 36.4 ± 0.8$^{e}$ |
| 0 | 1.0 | 0 | 2.0 | 40.1 ± 1.0$^{f}$ | 44.4 ± 1.1$^{f}$ | 40.1 ± 1.0$^{g}$ | 46.1 ± 1.0$^{g}$ |
| 0 | 1.5 | 0 | 2.0 | 56.6 ± 0.4$^{i}$ | 57.6 ± 0.7$^{i}$ | 59.6 ± 0.4$^{k}$ | 58.6 ± 0.7$^{i}$ |
| 0.25 | 0 | 0 | 2.0 | 46.9 ± 1.1$^{g}$ | 45.7 ± 1.1$^{f}$ | 46.6 ± 1.1$^{h}$ | 41.4 ± 1.1$^{f}$ |
| 0.25 | 0 | 3.0 | 0 | 58.4 ± 1.5$^{i}$ | 57.5± 0.9$^{i}$ | 54.4 ± 1.2$^{j}$ | 55.4 ±1.8$^{h}$ |

*20 replication/treatment; repeated thrice.
Means followed by the same letter within columns were not significantly different at $P < 0.05$.

not show any positive growth of the callus. However, the response of callus growth was different in different genotypes of rice tested. Similar response was also reported by many researchers by using different explant source that is, immature and mature embryos, leaf blade, coleoptile, meristematic cells and roots (Yan and Zhao, 1982; Koetje et al., 1989; Lee et al., 2002, 2004; Mandal et al., 2003).

Lee et al. (2002) reported that immature embryos were high responsive with embryogenic potential as compared with other explants. In most cases, 2,4-D as a strong synthetic auxin was sufficient to initiate and sustain embryogenic callus growth in rice and other monocots (Lee et al., 2002; Ozawa et al., 2003; Lin and Zhang, 2005; Ivarson et al., 2013; Pathi et al., 2013). The proliferated embryogenic calli were sub-cultured in various media for induction of high frequency somatic embryogenesis. L-proline at different concentrations was used for further growth of somatic embryos. High frequency proliferation of embryogenic calli was obtainined on MS medium supplemented with 1.0 to 2.0 mg/l Kn and 2.0 to 3.0 mg/l 2,4-D and 200 mg/l L-proline in all the four varieties tested. In some cases, the secondary somatic embryos were developed in subsequent subculture. The percentage of embryogenic calli proliferation were 82.4, 83.7, 88.4 and 84.4 in variety Khandagiri, Udayagiri, Swarna and Pratikhya respectively on MS basal medium supplemented with 3.0 mg/l 2,4-D, 2.0 mg/l Kinetin and 200 mg/l L-proline (Table 2). Inclusion of higher concentration of L-proline (400 mg/l) in the induction medium, the growth of calli was reduced. At lower concentrations (50 to 100 mg/l) of L-proline did not show any significant differences on embryonic callus growth and embryo development. The embryogenic calli grown in L-proline containing medium became more yellowish white in colouration and fragile in nature (Figure 1B). Auxin-induced somatic embryogenesis in presence of proline is well documented (Chowdhry et al., 1993). Free proline was suggested to act as an osmoticum, a nitrogen storage pool and source of $NADP^{+}$, necessary for rapidly growing embryos.

The mediation of the cellular redox potential resulted from proline accumulation likely had a significant effect on the flux through redox– sensitive biochemical pathways like the pentose phosphate pathway (Ghanti et al., 2009).

## Germination of somatic embryos

In addition to embryogenic callus formation, efficient regeneration also poses a major problem for transformation of *Indica* rice. The well developed group of embryos were transferred to various MS medium supplemented with growth regulators for germination. The medium devoid of growth regulators did not promote germination. The medium having 0.5 to 2.0 mg/l kinetin or BA along with 0.25 mg/l NAA and 50 mg/l adenine sulfate promote embryo germination. The germination frequency was varied in different genotypes. The maximum percentage of somatic embryo germination took place in medium supplemented with 2.0 mg/l kinetin, 0.25 mg/l

**Table 2.** Effect of different culture medium on proliferation of embryogenic calli of *O. sativa* varieties Khandagiri, Udayagiri, Swarna and Pratikhya after 8 weeks of subculture.

| MS + growth regulator (mg/l) | Percentage (%) of embryogenic calli [mean (average) ± SE]* | | | |
|---|---|---|---|---|
| | Khandagiri | Udayagiri | Swarna | Pratikhya |
| **0** | **0** | **0** | **0** | **0** |
| MS+ 2,4-D 1.0 + Kn 1.0 + 50 mg/l L-proline | 28.8 ± 0.9[a] | 27.3 ± 0.8[a] | 26.1 ± 0.6[a] | 28.9 ± 0.7[a] |
| MS+2,4-D 2.0 + Kn 1.0 + 100 mg/l L-proline | 37.9 ± 0.6[b] | 35.7 ± 0.7[b] | 35.3 ± 0.8[b] | 37.6 ± 0.8[b] |
| MS+ 2,4-D 2.0 + Kn 2.0 + 150 mg/l L-proline | 43.5 ± 1.0[c] | 41.2 ± 1.1[c] | 46.3 ± 1.2[c] | 43.6± 0.9[c] |
| MS + 2,4-D 2.0 + Kn 2.0 + 200 mg/l L-proline | 59.2 ± 0.8[e] | 57.25 ± 1.7[e] | 61.3 ± 1.0[e] | 62.5 ± 1.3[e] |
| MS + 2,4-D 3.0 + Kn 2.0 + 200mg/l L-proline | 82.4 ±1.4[g] | 83.7 ±1.4[g] | 88.4 ± 1.2[f] | 84.4 ±1.0[f] |
| MS+2,4-D 3.0+ Kn 2.0 + 50 mg/l L-proline | 62.9 ± 1.3[f] | 62.8 ±1.2[f] | 61.5 ± 1.1[e] | 63.1±0.9[e] |
| MS + 2,4-D 3.0 + Kn 2.0 + 100mg/l L-proline | 53.2 ± 1.8[d] | 51.5 ± 1.7[d] | 51.3 ± 1.7[d] | 52.5 ±1.2[d] |
| MS + 2,4-D 2.0 + Kn 2.0 + 200mg/l L-proline | 38.8 ± 0.7[b] | 37.3 ± 1.0[b] | 36.1 ±0.9[b] | 38.9 ±0.7[b] |

*20 replication/treatment; repeated thrice.
Means followed by the same letter within columns were not significantly different at P < 0.05.

**Table 3.** Effect of growth regulators on germination of somatic embryos of *O. sativa* varieties Khandagiri, Udayagiri, Swarna and Pratikhya after 4 weeks of culture.

| MS + Growth regulators (mg/l) | Percentage (%) of somatic embryo germinated (mean ± SE)* | | | |
|---|---|---|---|---|
| | Khandagiri | Udayagiri | Swarna | Pratikhya |
| **0** | **0** | **0** | **0** | **0** |
| MS + Kn 0.5 + NAA 0.25 + Ads 50 mg/l | 15.2 ± 0.7[a] | 25.2 ± 1.0[a] | 15.5±0.9[a] | 13.7 ± 0.8[a] |
| MS + Kn 1.0+ NAA 0.25 + Ads 50 mg/l | 33.8 ±0.9[b] | 32.8 ± 0.7[b] | 27.8±1.0[b] | 26.3 ± 0.6[b] |
| MS + Kn 1.5 + NAA 0.25 + Ads 50 mg/l | 57.2 ± 1.3[e] | 48.7 ± 1.1[e] | 48.2 ±1.3[d] | 46.5 ± 1.2[c] |
| MS + Kn 2.0 + NAA 0.25 + Ads 50 mg/l | 72.5 ±1.5[f] | 67.5 ±0.6[g] | 62.2±1.2[f] | 58.5 ± 1.3[d] |
| MS + BA 1.0 + NAA 0.25 + Ads 50 mg/l | 42.3 ± 1.6[c] | 38.8 ±0.7[c] | 36.6±1.0[c] | 47.5 ± 1.5[c] |
| MS + BAP 2.0 + NAA 0.25 + Ads 50 mg/l | 48.5 ± 1.1[d] | 41 ±0.9[d] | 45.4±1.2[d] | 46.3 ± 1.2[c] |
| MS + BA 2.0 + Kn 1.0 + NAA 0.25 + Ads 50 mg/l | 43.35 ±1.2[c] | 57.8 ±1.8[f] | 52.2±1.3[e] | 62.7 ± 1.4[e] |

*20 replication/treatment; repeated thrice.
Means followed by the same letter within columns were not significantly different at P < 0.05.

NAA and 50 mg/l adenine sulfate within 4 weeks of culture (Table 3). About 60 to 70% of embryos were germinated in different culture medium (Figure 1C and D). Further, the germinated embryos were developed into complete plantlets. With increase of NAA concentrations from 0.25 to 0.5 mg/l in the culture medium, the germination frequency was lower down. Extensive research has been conducted to improve the capacity of plantlet regeneration by manipulating the important factors within regeneration medium, such as carbohydrate source (Lee et al., 2002), nitrogen source (Grimes and Hodges, 1990), polyamines (Bajaj and Rajam, 1995, 1996), amino acids such as proline and tryptophan (Ozawa and Komamine, 1989; Chowdhry et al., 1993), and plant growth regulators (Kavi Kishor, 1987).

The medium supplemented with BA + NAA also promote embryo development and germination frequency but less than the Kn + NAA. Further, the germinated plantlets were transferred to sterile distilled water for a week and kept in the culture room for acclimatization (Figure 1E). After one week, the plants were planted in the pots and transferred to the greenhouse with 70% humidity. About 80% of plants survived in the greenhouse condition (Figure 1F).

## Conclusion

Plant growth regulators and physiological status of the explants play a critical role in the control of growth and morphogenesis. It may be concluded that genotypic differences strongly influence on embryogenic callus formation and plant regeneration potential. The results suggest that embryonic explants are very good source material for efficient *in vitro* plant regeneration. The high frequency plant regeneration through somatic embryogenesis will be suitable for genetic transformation study of upland and lowland rice varieties.

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**Figure 1.** *In vitro* somatic embryogenesis of different varieties of rice (*O. Sativa* L.).
A. Induction of somatic embryogenesis of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 3.0 mg/l 2,4-D and 1.0 mg/l Kinetin (bar = 10 mm).
B. Induction of somatic embryogenesis of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 3.0 mg/l 2,4-D and 2.0 mg/l Kinetin and 200 mg/l L-proline (bar = 50 mm).
C. Germination of somatic embryos of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 2.0 mg/l Kinetin, 0.25 mg/l NAA and 50 mg/l adenine sulphate (bar = 25 mm).
D. Germination of somatic embryos of *O. sativa* varieties Khandagiri after 4 weeks of culture on MS medium supplemented with 2.0 mg/l Kinetin, 0.25 mg/l NAA and 50 mg/l adenine sulphate (bar = 25 mm).
E. Somatic embryo derived plantlets were transferred to distilled water for primary hardening and acclimatization.
F. Germinated plantlets of *O. Sativa* varieties Khandagiri grown in the greenhouse (bar = 1.0 cm).

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